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# Memoirs of the Department of Agriculture in India

## STUDIES ON THE ROOT NODULE ORGANISM OF THE LEGUMINOUS PLANTS

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BY

N. V. JOSHI, B.A., M.Sc., L. Ag

*First Assistant to the Imperial Agricultural Bacteriologist*

AGRICULTURAL RESEARCH INSTITUTE, PUSA

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# STUDIES ON THE ROOT NODULE ORGANISM OF THE LEGUMINOUS PLANTS.

BY

N. V. JOSHI, B.A., M.S.c., L.Ag.,

*First Assistant to the Imperial Agricultural Bacteriologist.*

[Received for publication on 16th September, 1919.]

It has long been known that leguminous crops somehow enrich the land, and agriculturists have made use of this knowledge in practice by growing leguminous crops for restoring the exhausted soils to their normal condition or reclaiming barren lands. It is also a familiar fact that the roots of leguminous plants contain nodular swellings or excrescences known as root nodules or tubercles. According to the earliest recorded observations, these root nodules were looked upon as mere modifications of the normal roots or pathological outgrowths due to some disease. Although careful microscopic examination by later investigators revealed the presence of micro-organisms in the cells of the nodules, and although these micro-organisms were assumed to be causative agents of nodule formation, the nodules were still regarded as malformations caused by a parasitic organism. There was no suggestion of a connection between the micro-organisms contained in the root nodules of legumes and the reputed soil-enriching qualities of the legumes. The fact that such connection does exist was not known until comparatively recently, the first demonstrable proof of the existing relation having been given by Hellriegel and Wilfarth[1] between 1886–1888. These authors showed that the legume tubercles were caused by bacterial infection, that this infection is bene cial rather than harmful to the host plant, since it is the means of providin the latter with combined nitrogen, which is obtained through bacterial activity from the free nitrogen of the atmosphere. This discovery led several investigators in various parts of the world to study the bacteria in the nodules of leguminous plants. The results of their work have confirmed the above

[1] Hellriegel & Wilfarth. "Zeits. des Vereins f.d. Rubenzucker-Industries," 1888.

statements of Hellriegel and Wilfarth, establishing the symbiotic relationship between bacteria and legumes in the fixation of atmospheric nitrogen. In spite of numerous investigations on the organism, however, the literature on the subject is full of contradictory statements, no general agreement having been reached on many important points which are under dispute and therefore undecided as yet. Thus opinion is still divided on the question of nitrogen fixation by the nodule organism, independent of the plant. Some authors claim to have demonstrated large gains of nitrogen in laboratory cultures, while others obtained no gain or so slight a gain of nitrogen as to be within the limits of experimental error. Erwin Smith,[1] on reviewing the subject of nitrogen fixation by the root nodule organisms, makes the following observations :— "The whole subject of the storage of free nitrogen by this organism in flask cultures and in the plant itself ought to be worked over again carefully by the bacteriologist and the chemist. Possibly root nodules are only indicators of a fixation of nitrogen which actually takes place in the soil. Certainly it should be determined whether *Bact. leguminosarum* (Frank) is able to fix nitrogen outside of the plant in agricultural soil, both sterilized and unsterilized." It may be mentioned here in passing that the questions whether or to what extent nitrogen fixation by the organism independent of the plant can take place in soil and if so what are the requisite conditions suitable for the purpose, are of great practical importance because on their solution depends the simplification of the problem of nitrogenous manuring which, as recently pointed out by Hutchinson,[2] is of great interest especially in a country like India which does not import nitrogenous fertilizers.

Then again the question whether there is a single species of legume nodule organism or a separate race of bacteria for each kind of plant is still not definitely settled. Some hold that there are as many different kinds of organisms as the leguminous plants, while others maintain that there is only a single species. Between these two opposite extremes are some who favour the idea of a limited number of species although not agreeing as to the exact number.

The isolation of the organism from the soil is also a debatable point. Some have claimed the isolation of the organisms directly from the soil but these claims are not supported by others and are therefore not generally accepted. Thus Russell[3] writing on the subject gives it as his opinion that "none of these organisms, however, could be found in the soil, nor indeed has any one yet succeeded in finding them there although their existence cannot be doubted."

[1] Smith, E. F. "Bacteria in relation to plant diseases," Vol. II, p. 99.
[2] Hutchinson, C. M. *Journ. of Agr. India*, Vol. XIV, pp. 203–214.
[3] Russell, E. J. "Soil conditions and plant growth," 3rd ed., p. 128, 1917.

Different views are again expressed on the mechanism of nitrogen fixation or as to how the organism is enabled to fix atmospheric nitrogen and what is the first product of synthesis.

Besides these fundamental questions of practical importance, there are a number of other minor issues on which opinion is not unanimous. This state of affairs is partly due to contradictory results obtained by different investigators working under diverse conditions and each one of them making general deductions based on work which was carried out under a particular set of conditions. Although in a complicated problem involving several factors this result is not unexpected, it shows the necessity of a still greater amount of work under conditions which can be controlled at will, without which it would not be possible to explain or reconcile the extreme views held by different workers.

The present writer had to demonstrate some work in connection with this subject to the students in this laboratory, and the opportunity was taken to investigate the question of species by cross-inoculation of the organism from root nodules of one plant on another, and the nitrogen-fixing power of the organism independent of the plant under laboratory conditions. In the course of this work some interesting results were obtained and so further work on the subject was done during the last few years. It is not claimed that the work is in any way complete. The chief object in putting together the results obtained so far is, however, to elicit criticism of methods employed, and in the light of that criticism to know the exact position reached with regard to the subject.

*Medium.* The first necessity in respect of work with this root nodule organism is the choice of some suitable medium. The culture medium upon which various strains of the root nodule organism were isolated and grown in this laboratory had the following composition :—

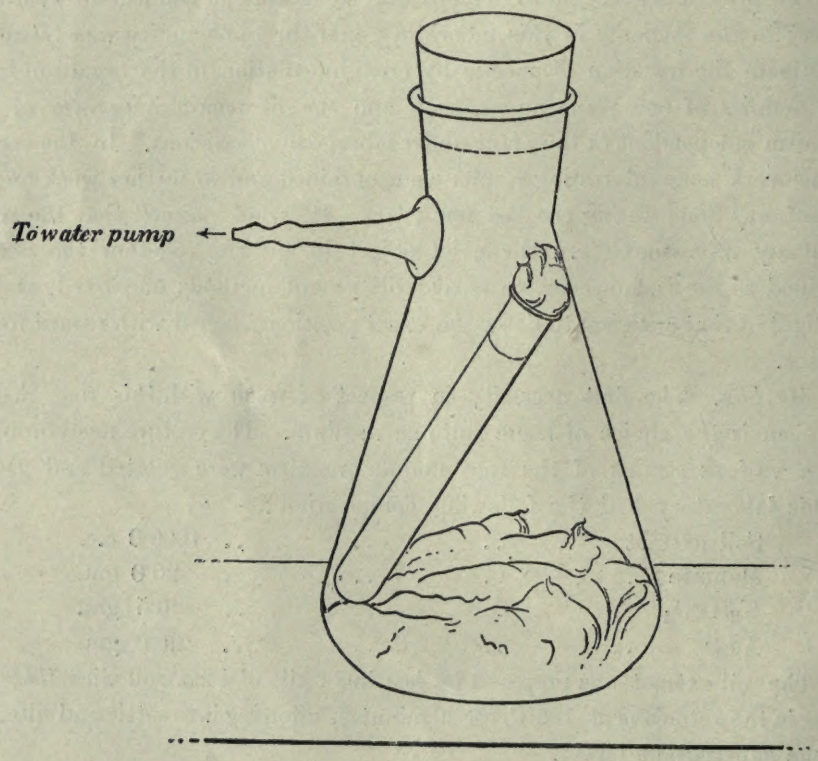
| | |
|---|---|
| Soil extract .. .. .. | 1000·0 c.c. |
| Mannite .. .. .. .. | 20·0 gm. |
| $K_2HPO_4$ .. .. .. .. | 0·5 gm. |
| Agar .. .. .. .. | 20·0 gm. |

The soil extract was prepared by heating 1 kilo of Pusa soil with 1500 c.c. water in the autoclave at 120°C. for 20 minutes, allowing it to settle and filtering off the supernatant liquid.

A comparison of this medium with media used by others will be found later on; but it may be remarked here that this medium proved very satisfactory for the purpose and gave a luxuriant growth of the nodule organisms isolated from various species of the leguminous plants.

*Isolation.* The next consideration is the method of isolation. In isolating the organism the following method was used. The roots of the plant from which organisms were to be isolated, were washed free from soil; sound nodules were selected and cut off from the roots, leaving a few millimetres of the root on both sides so as to render it easier to handle the nodule with forceps. It was also considered necessary to leave portions of root attached to the nodule, as otherwise it was feared that the contents of the nodule would be injured if the sterilizing fluid, to be used subsequently, were to penetrate into the interior through the slight wound caused by breaking off the nodule altogether.

The sterilization of the nodules was done according to the method recommended by Hutchinson and Miller[1] for seeds, but instead of their elaborate and cumbrous apparatus a simple but equally efficient one, as shown in figure below




was used. The nodules were carefully washed in distilled water and dropped in a sterilized test tube, containing a few c.c. of warm mercuric

[1] Hutchinson & Miller. *Centralblatt. f. Bakt.*, II, Abt. 30, p. 526.

chloride (temperature 40°C.). The test-tube was then replugged with cotton wool and placed in a filtering flask fitted with a rubber cork. It may be mentioned that three or four test tubes can be put in the flask in case one has to isolate nodule organisms from more than one kind of plants. The flask was afterwards connected to the filter pump and the air exhausted till the solution began to boil. In this way all air bubbles present on the surface of the nodule are withdrawn, and on admission of air by disconnecting the pump the nodules sink to the bottom of the test-tube and the disinfectant solution is able to act on all portions of the nodule.

Sterilization was allowed to proceed for two minutes after which the tube containing the nodules was taken out. The mercuric chloride was poured out and the nodules were washed three to six times with sterile distilled water. The nodules were then crushed inside the tube by a sterile glass rod. Two platinum loopfuls of the cloudy suspension were transferred into a soil-extract-mannite-agar tube, and two loopfuls from the first inoculated tube of agar into a second tube, and two loopfuls again from the second to the third. The contents of the tubes were separately poured into three Petri dishes and the plates incubated at 30°C. The colonies usually began to appear after three or four days—in some cases after five or six days—and were ready for inoculation in about a week. Transfers were made from individual colonies on slants of the same agar and stock cultures preserved by repeated transfers. All the organisms were isolated from the roots of the plants by the above method. No foreign organisms were noticed on plates, the method for sterilizing the outside surface being strictly adhered to.

At first several strains of the nodule organism from cow-pea (*Vigna catjang*) were isolated in order to be familiar with the cultural characteristics of the organism and to find out whether there was any difference noticeable. As the cultural characteristics of the different strains agreed very well among themselves as well as with the published descriptions, it was proposed to use two of them for cross-inoculation and to test their nitrogen-fixing power.

For cross-inoculation the plants which are to be inoculated may be grown in (1) nutrient solution contained in flasks or test-tubes, (2) in agar contained in flasks or in test tubes, which is advocated by Garman,[1] or (3) in sand in pots.

The first two methods though convenient were not found very successful in this laboratory on previous occasions, and were therefore thought unsuitable for cross-inoculation. The third method is rather difficult, as it is necessary to

[1] Garman & Didlake, *Ky. Agr. Expt. Sta. Bull.* 184.

maintain the sterile conditions not only at the time of starting but throughout the growing period. However it was proposed to try the glazed earthenware pots of special design already described by Hutchinson.[1] The pots were filled with coarse sand which was previously ignited to deprive it of its organic nitrogen and the pots were sterilized in the autoclave at 130°C. for half an hour on two days. Seeds of *math* or *matki* (*Phaseolus aconitifolius*) were sterilized by the same method as was used in the case of nodules, sown by means of sterile forceps and watered with sterile water through the porous candle. After they had germinated it was proposed to supply all the ingredients except nitrogen to all the pots and to leave one pot uninoculated as control and to inoculate separately the others with an emulsion of the strains of nodule organisms from cow-pea. The emulsion was made in a solution containing glucose and Pot. phosphate to which some lime was added. The emulsion was added to the pots by means of a tube one inch below the surface, so as not to contaminate the surface which was kept dry. The cover was removed after the plants had made some growth. It is expected that under sterile conditions the control plants receiving all the other plant foods except nitrogen but no inoculum ought to die after some time being starved of nitrogen, while those inoculated should survive getting their supply of nitrogen through the organism if the inoculation should prove successful as evidenced by nodule formation.

The experiment lasted for six weeks from 5th September, 1915, to 20th October, 1915, at which time the plants in the control pot began to wilt one after another. The plants from both pots were therefore uprooted and photographed. The accompanying photograph (Plate I) shows the result. The effect of inoculation is obvious; the roots of the uninoculated plants have not grown in length, while those of the inoculated show nodules and have developed to a greater length showing that they received a stimulus by the inoculation. The fact that plants in the control pot were starved shows that the specially designed pots used in this experiment were suitable for the work and that it was possible to maintain sterile conditions throughout the growth of the plant, at least sufficiently sterile to exclude the nodule organism.

*Nitrogen fixation.* It was now proposed to re-isolate the organism from the nodules of plants in one pot and compare its nitrogen fixing in flask cultures with that of the cultures of original organisms used for inoculation.

Two strains from cow-pea and one isolated from the inoculated *Phaseolus* were separately inoculated in duplicate flasks each containing 100 c.c. soil extract to which was added 1 per cent. mannite and 0·05 per cent $K_2HPO_4$.

[1] Hutchinson. *Memoirs, Dept. Agr. India, Bact. Series*, I, p. 18 (1910-11).

PLATE I.

Nitrogen was estimated from these after 10 days. The results are given in the following table.

It is evident from the results set down that these strains of the organisms two of which were found to have the nodule-forming power, the third one being re-isolated from the nodules so formed, do not show any considerable nitrogen fixation in flask cultures.

TABLE I.

| | Cow-pea 1 | Cow-pea 2 | *Pha seolus.* |
|---|---|---|---|
| Mgm. nitrogen found in duplicate culture flasks .. | 6·2 | 5·8 | 5·9 |
| | 5·8 | 5·4 | 5·7 |
| Average nitrogen fixed in mgm. .. .. | 6·0 | 5·6 | 5·8 |
| Mgm. nitrogen found in control .. .. | 4·8 | 4·8 | 4·8 |
| Total gain in nitrogen mgm. .. | 1·2 | 0·8 | 1·0 |

A second experiment was arranged for a greater number of organisms to be cross-inoculated on pea (*Pisum sativum*), *math* (*Phaseolus aconitifolius*), and gram (*Cicer arietinum*). The method of filling of the pots and isolation of nodules and inoculation was the same as in the first experiment. After four weeks the control plants began to show signs of dwindling when all the plants were uprooted and examined for nodule formation.

The results are given in the following table wherein

\+ = presence of nodule.

− = absence of nodule.

TABLE II.

| | Pea | Math | Gram |
|---|---|---|---|
| Control .. .. .. .. .. .. | − | − | − |
| Nodule organism from Cow-pea (*Vigna catjang*) No. 1 .. .. | + | + | − |
| „ „ from Cow-pea (*Vigna catjang*) No. 2 .. .. | + | + | − |
| „ „ from Arhar (*Cajanus indicus*) .. .. | − | + | − |
| „ „ from Dhaincha (*Sesbania aculeata*) .. .. | − | − | − |
| „ „ from Gokarn (*Clitoria ternatea*) .. .. | − | − | − |

When the roots were being examined it was at once noticeable that although some of the inoculated plants had no nodules on them, still their roots appeared

much more vigorous than those of the uninoculated control, the inoculation of the organism appearing to exercise a stimulating effect. This is well illustrated in photographs some of which are reproduced (Plates II and III). All the strains were tested for their nitrogen-fixing power in culture flasks. The highest amount of nitrogen fixed by any one of these strains was 2·0 mgm. in 100 c.c. of culture solution. On an average it amounted to 1·4 mgm. nitrogen only per 100 c.c. culture solution.

A third experiment was arranged to see whether the results could be confirmed by reinoculation of some of the organisms on pea. The accompanying photograph (Plate IV) shows the effect on growth of the plant; and Table III gives the results wherein again

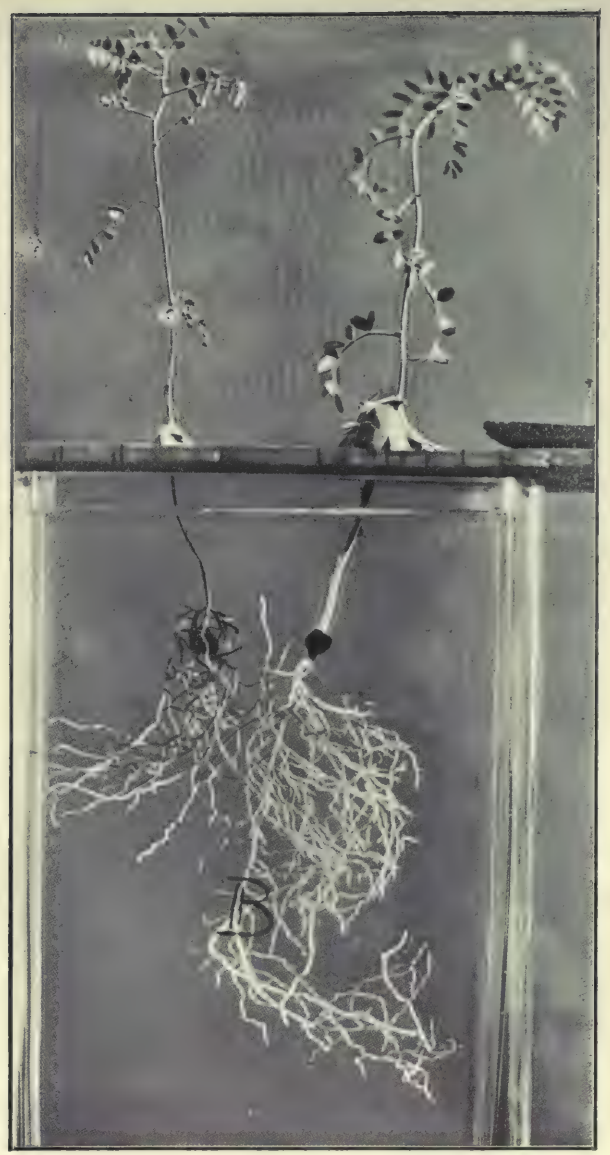
\+ = presence of nodules.
− = absence of nodules.

TABLE III.

| | Pea |
|---|---|
| Control .. .. .. .. .. .. .. | − |
| Root nodule organisms from Sann-hemp (*Crotalaria juncea*) .. .. | + |
| „ „ „ from Cow-pea (*Vigna catjang*) .. .. | + |
| „ „ „ from Gokarn (*Clitoria ternatea*) .. .. | − |

It is evident that *Vigna* × pea and *Clitoria* × pea give the same results as in the former case. *Crotalaria juncea* × pea gives a positive result.

The results of the latter two experiments show that substantial increase in growth of roots and of the whole plant results from inoculation of root nodule organisms although not always accompanied by nodule formation. In the case of those plants which showed nodules, the increased growth may be accounted for by the nitrogen supplied through the activity of the organisms in the nodule. In those cases, however, where there was a complete absence of nodules it may be suggested (1) that the nitrogen might be fixed by the organisms in the sand independent of the plant, or (2) that the organisms might be present in roots of the plants without formation of nodules due either to lower virulence of the organism or the slow reaction of the plant to the stimulus of the organisms. A search was, therefore, made by cutting sections of such roots after sterilizing the outside. Although many sections of the roots were examined no organisms could be detected in them. It must be admitted that the search for the organism inside the tissues of roots without nodules cannot be exhaustive on account of the impossibility of examining

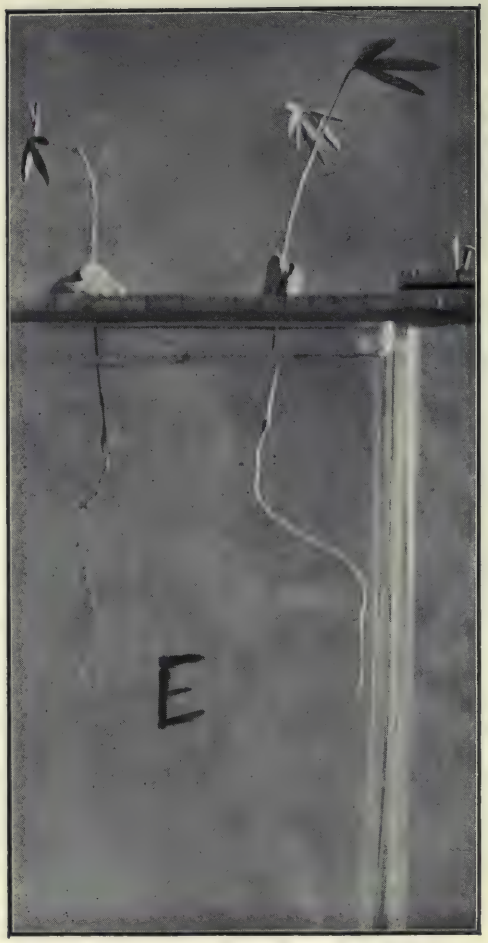


Uninoculated. Inoculated with Cow-pea Radicicola

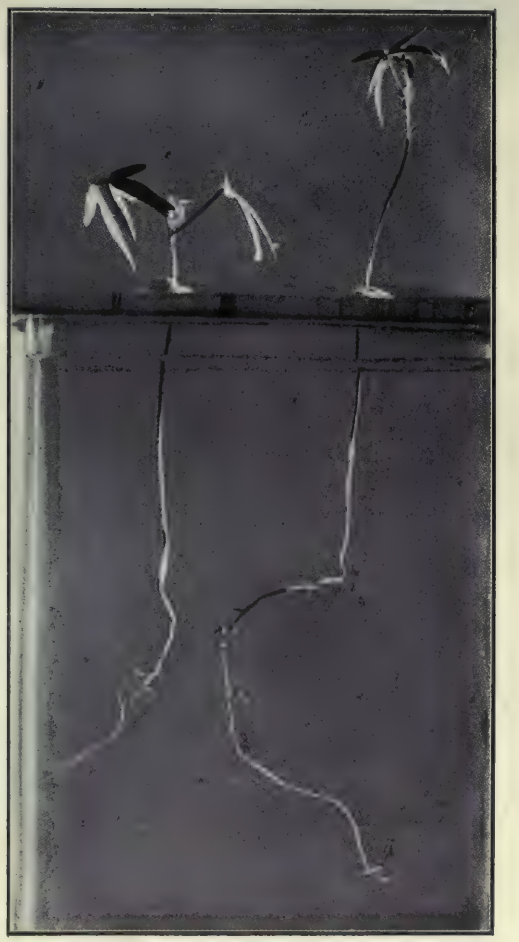
Uninoculated. Inoculateh with Arhar Radicicola.

Gram (*Cicer arietinum.*)

PLATE II.




Uninoculated. Inoculated with Arhar Radicicola.



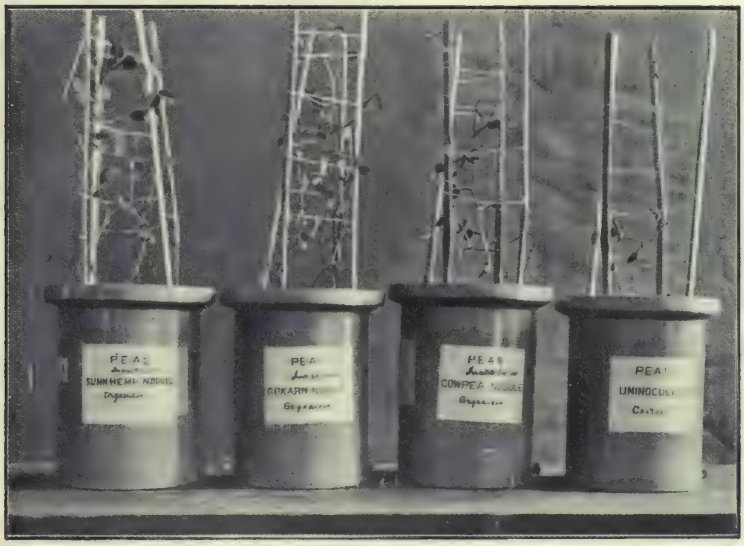
Uninoculatd. Inoculated with Cow-pea Radicicola.

Math (*Phaseolus aconitifolius.*)

PLATE III.

PLATE IV.

Peas.



Inoculated with | Control.

Sann-hemp Radicicola | Gokarn Radicicola | Cow-pea Radicicola | *Uninoculated.*

the whole root in section. Therefore, although we have still to recognize the possibility of finding the nodule-forming organism inside the root tissue in the complete absence of nodules on the roots, we are inclined to believe such a contingency to be a very remote one.

In order to examine the feasibility of the first suggestion, nitrogen was determined in sand from the uninoculated and the inoculated pot but no appreciable gain could be found, but this is not a satisfactory test as the sand may not show any increase in nitrogen and yet the plant may have already assimilated a greater part of it. It was proposed, therefore, to examine the question by further experiments on new lines based on the hypothetical assumption that the nitrogen is fixed in the sand and utilized by the plant. The logical conclusions, if this assumption is taken as correct, are as follows: (1) The nitrogen thus fixed must be equally available to any kind of plant whether belonging to leguminosæ or not; (2) also if one kind of nitrogen-fixing organism could bring about increased growth another organism like azotobacter which has a higher power of fixing nitrogen in the flask cultures as compared to the root nodule organism may be able to show the same effect; (3) if a part of the total quantity of nitrogen fixed by the organism is assimilated by the plants without the intervention of the nitrifying bacteria it is very likely to be that part which is soluble and as such filtrable through a porous cylinder. Hence if the organism be growing in a porous cylinder placed in the pot containing the sand, the plant may be able to get the soluble nitrogenous material which will pass through the porous cylinder.

The following plan of treatment was therefore prepared with the object of finding out a solution of questions involved in the above arguments. Potassium nitrate was added to pot B with a view to observe its effect on the growth of plants for comparison with other treatments.

*Scheme of experiments regarding inoculation of nodule organisms in different kinds of leguminous and graminaceous plants, October* 1917 *to January* 1918, *and October* 1918 *to February* 1919.

A. Control (Sterilized burnt sand + *Glucose* and *Pot. Phosphate* in sterile solution).

B. Control + $KNO_3$ added as sterile solution.

C. Control + Inoculated with azotobacter (added as suspension made in sterile solution).

D. Control + Inoculated with pea nodule organism (added as suspension made in sterile solution).

E. Control + Inoculated with *Phaseolus* nodule organism (added as suspension made in sterile solution).

F. Control + Inoculated with gram nodule organism (added as suspension made in sterile solution).

G. Control + Mixture of cultures of the above three nodule organisms in a porous pot.*

It was proposed to grow the following six crops, three of which belong to the leguminosæ and three to graminaceæ :—

(1) Pea (*Pisum sativum*).

(2) Math (*Phaseolus aconitifolius*).

(3) Gram (*Cicer arietinum*).

(4) Maize (*Zea Mays*).

(5) Oats (*Avena sativa*).

(6) Wheat (*Triticum vulgare*).

The same experiment was repeated in October 1918, and lasted up to February 1919. The results of these two experiments were similar.

At the close of each of these experiments examination of the roots was made with a view to ascertain the presence or absence of nodules on roots, of nodule organisms inside the roots, of nitrifying organisms in the culture pots. Quantitative determinations were also made of dry matter of the crop and its nitrogen content in each of the pots. The results of these will be found in the discussion that follows.

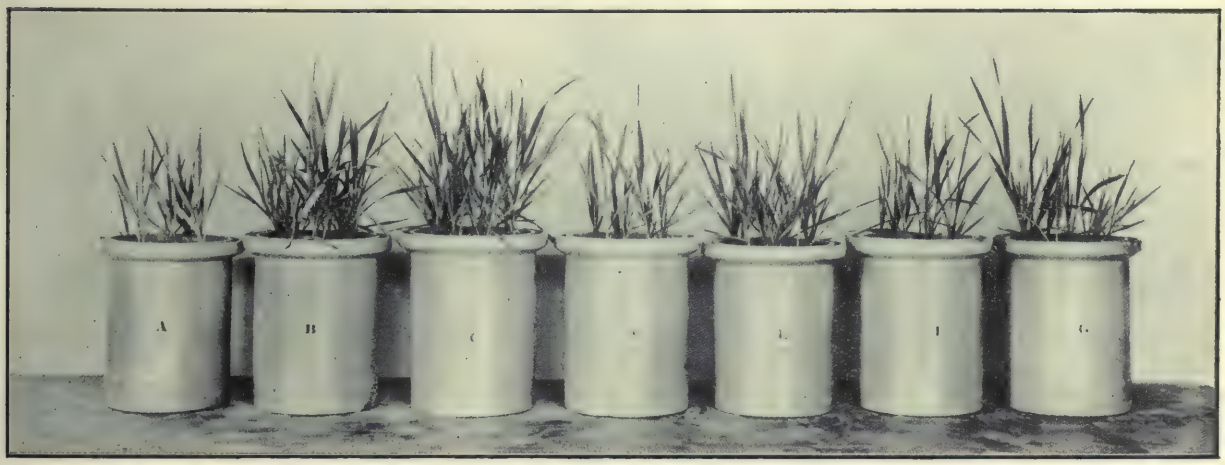
It may be observed in general that the results of both the experiments were similar and fully confirmed those obtained in previous experiments. The observations recorded below apply therefore equally to either of these two experiments. The effect of various treatments on the growth of plants is very well shown by photographs, some of which are reproduced here being selected as typical (Plates V—IX). These were taken just before the time of wilting of control plants.

It was at once evident that the plants which received nitrates were the best. The leguminous plants which were inoculated with their own organisms very nearly approach those receiving $KNO_3$, suggesting the obvious inference that the nodule organism can supply all the nitrogen that is needed by the plant. Next in order come the cross-inoculated, the azotobacter-inoculated, and those in which the organisms were growing in the porous cylinder. It is not possible to distinguish accurately between these as the effects are very close to one another ; and some kind of treatment shows a better effect in one series than in another. Although no particular treatment stands out as

* It is presumed that the plants have not come in direct contact with the organisms in the case.

Peas (*Pisum sativum.*)

Gram (*Cicer arietinum.*)




Oats (*Avena sativa.*)

Letters on pots indicate treatment:—A, Control; B, Potassium nitrate; C, azotobacter; D, Pea radicicola; E, *Phaseolus* radicicola; F, Gram radicicola; G, Pea, *Phaseolus* and Gram radicicola in porous pot.

Pea Gram *Phaseolus.*

With Potassium Nitrate.

Pea Gram *Phaseolus*

Inoculated with

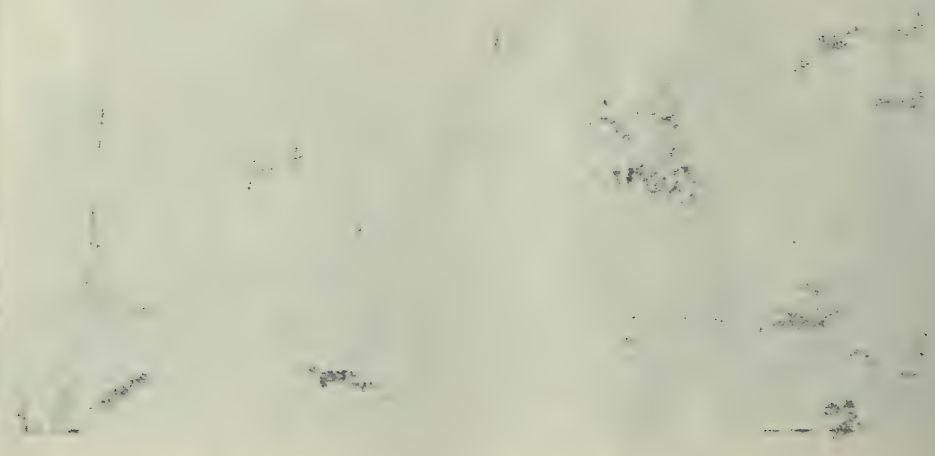
Pea Radicicola — Gram Radicicola — *Phaseolus.* Radicicola.

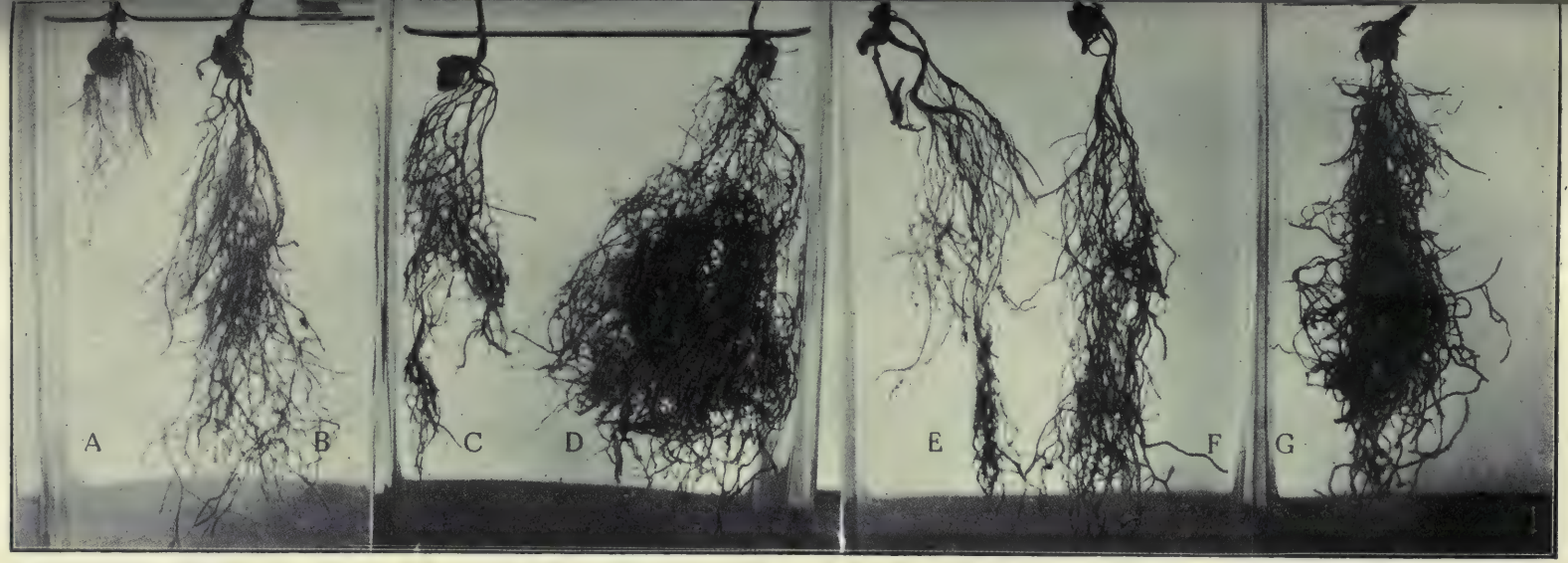
Pea (*Pisum sativum.*)

Math (*Phaseolus aconitifolius.*)

Oats (*Avena sativa.*)

Letters on pots indicate treatment:—A, Control; B, Potassium nitrate; C, azotobacter; D, Pea radicicola; E, *Phaseolus* radicicola; F, Gram radicicola; G, Pea, *Phaseolus* and Gram radicicola in porous pot.






Roots of Peas (*Pisum sativum.*), 1917-18.

Roots of Gram (*Cicer arietinum.*) 1917-18.

Letters indicate treatment:—A, Control; B, Potassium nitrate; C, Azotobacter; D, Pea radicicola; E, *Phaseolus* radicicola; F, Gram radicicola; G, Pea, *Phaseolus* and Gram radicicola in porous pot.

superior in all the series, they are all better than the controls where the plants show poor growth. The effect of nodule organisms on maize, oats and wheat is also marked showing that graminaceous plants also have benefited to a certain extent from inoculation of this organism. Of course no nodules were found on the roots of any of the graminaceous plants.

Results of subsequent examination of the root systems with regard to the presence or absence of nodules are set out in the following table :—

TABLE IV.

| | Pea | Math | Gram | Maize | Oats | Wheat |
|---|---|---|---|---|---|---|
| A | − | − | − | − | − | − |
| B | − | − | − | − | − | − |
| C | − | − | − | − | − | − |
| D | + | + | − | − | − | − |
| E | + | + | − | − | − | − |
| F | − | − | + | − | − | − |
| G | ∓* | − | − | − | − | − |

In the first three series, A, B, C, no nodule formation was expected and it is also not found showing that infection by outside nodule organisms has not taken place. Further tests for outside infection were carried out at the close of the experiment.

Pea × *Phaseolus* gives positive results. The same is the case with the organism *Phaseolus* × pea.

Pea organism × gram, and *Phaseolus* organism × gram give negative results.

Gram organism × pea, and gram organism × *Phaseolus* give negative results.

In the last series G with porous pots also no nodules were found except once.

These results, taken with the previous ones already noted, raise the question, what is the significance of the fact that inoculation of nodule organisms isolated from different plants leads to nodule formation in some cases and does not in others ? Does this mean that there are so many distinct species ?

* In one of the experiments the result was positive, possibly due to some inoculum dropping outside the porous pot.

If the test of nodule formation is applied to divide the organism into species, the number of such species will depend on the experiments carried out and results obtained by one investigator, and may perhaps be constant so far as the investigations of one worker are concerned. When, however, we try to compare the results of previous investigators, we find that no common agreement can be reached. Thus to take only the three most recent attempts to divide the nodule organism into species we get the following results :—

Garman and Didlake[1] divide the organism into six kinds which they call species. Burril and Hansen[2] divide the organism into eleven kinds and although they call them varieties of one species, six of their varieties are the same as the six species of Garman and Didlake. Burril and Hansen disregard the work of previous authors who claim to have produced cross-inoculations, notably of Laurent,[3] Mazé,[4] Moore,[5] and Kellerman,[6] assigning faulty technique as their reason for doing so.

Koch and Butler[7] have investigated four of the species cited by Garman and Didlake and have confirmed their results.

If we compare the results obtained in our investigations we, however, find no confirmation. Thus the six species proposed by Garman and Didlake, naming them by the prominent plant in the group of plants, are as follows :—

1. *Trifolium* or clover group.
2. Alfalfa group.
3. Cow-pea group.
4. Pea-vetch group.
5. Soybean group.
6. *Phaseolus* group.

These investigators have found that these species of nodule organism do not cross-inoculate *i.e.*, do not produce nodules when organisms from plants of one group are inoculated on plants of another group. They do not say anything as to whether the plants derived any benefit from cross-inoculation as compared with the uninoculated control plants. In fact their experiments were not arranged for that purpose but were done with the sole object of determining whether nodules are formed by cross-inoculation. From our results it is evident that we will have to eliminate 3, 4 and 6 species and group

[1] Garman & Didlake. *Ky. Agr. Expt. Sta. Bull.* 184 (1914).
[2] Burril & Hansen. *Ill. Agr. Expt. Sta. Bull.* 202 (1917).
[3] Laurent. *Compt. Rend. Acad. Sci.*, 111, p. 754 & *Bot. Centr.* (1891).
[4] Mazé. *Ann. Inst. Past.*, 13, p. 145 (1899).
[5] Moore. *U. S. Dept. Agr. Bur. Plant Indus. Bull.* 71 (1905).
[6] Kellerman. *Centr. f. Bakt.*, II Abt., 34, 42-50 (1912).
[7] Koch & Butler. *Soil Science*, VI, p. 397.

them together as one, since the results of cross-inoculation in our experiments were as follows :—

| | |
|---|---|
| Cow-pea × *Phaseolus* | positive |
| Pea × *Phaseolus* | positive |
| Cow-pea × pea | positive |
| *Phaseolus* × pea | positive |

We may have to add one more species as the organism from *Cicer arietinum* has not been found to cross-inoculate *Phaseolus* and pea and *vice versa.*

It will be realized, therefore, that no division of the organism is likely to be permanent unless there is some agreement among different authors ; and although one may be disposed to disregard the work of previous authors as faulty in technique, it is not possible to rely upon the evidence furnished by one investigator alone, without confirmation by others. If we take into consideration the combined results of all workers the species will most probably be limited to one.

It may be observed in this connection that the question whether there are more species than one of this organism has been raised in connection with the unsuccessful attempts to grow alfalfa and soybean in some parts of the world and especially in places where no legumes were grown before, and naturally the first idea that suggested itself was that the species of the root nodule organism were different. It must be remembered, however, that along with the unsuccessful attempts there were equally successful ones. No great difficulty was experienced in introducing lucerne, soybean or groundnut, in various parts of India, wherever these crops were grown for the first time, showing that in soils where some leguminous crops were previously grown the organisms are well adapted to inoculate new crops. As a matter of fact attempts to grow better leguminous crops with commercial cultures of nitrogen-fixing organism like *nitragin* and nitrobacterine, have not been successful in India. This shows that the organisms already present in Indian soils are efficient for the purpose and need not be replaced by those from outside. We are not justified, therefore, in dividing organisms into species, simply on account of failure in establishing crops like alfalfa in some cases which can be explained on other grounds as well.

In considering classification of bacteria we have often to note several characteristics and note their constancy or otherwise. It is impossible to classify them by simply relying upon any casual observation of the absence of one characteristic when all the other characteristics are constant. Thus it is known, for instance, that many Chromogenic bacteria sometimes lose their power of forming pigments by exposure to light. The same effect also results

from exposure to ultra-violet rays or sometimes even by repeated cultures on the same medium for a long time. Others again acquire or lose a particular characteristic by being grown in a medium containing certain chemical compounds. Thus it has been shown by Cecil Revis[1] that in the presence of certain sugars, polyhydric alcohols, Malachite green and brilliant green, organisms of the typical *B. coli* type and *B. acidi lactici* type can undergo a change in physiological activity, resulting in an entire and permanent loss of gas-producing power. Similar changes may, therefore, be expected in the case of other organisms also. The organisms in the nodules by their peculiar environment, that of being in close association with and getting all their nutrients from their host plant, are particularly subject to such influence of chemical compounds present in the root cell-sap of leguminous plants. Even minute quantities of these compounds may be sufficient to bring about profound changes in their physiological activity. Therefore, a variability of these organisms from one kind of plant to another may well be expected, especially if they are recently isolated. The remarkable power of discrimination in entering the particular species of plant to which the organism is accustomed can, therefore, be explained by supposing that the particular strain is derived originally from the single cell most influenced in this way[2]. Hence, the really remarkable fact is that, in spite of such influences, the different strains of this organism are so much alike. As a matter of fact the several strains of the organism show such a wonderful constancy of all the characteristics, except the occasional absence of nodule formation noticed in cross-inoculation experiments, and so widely do they differ from any other species of bacteria that we are disposed to regard them all as belonging to one species.

Even as regards the non-formation of nodules several investigators[3] have shown that it is possible to induce nodule formation by cross-inoculations which are known not to occur naturally. This success is claimed to have been achieved by previously cultivating the organism on suitable media before cross-inoculation. It seems, therefore, that the possibility of accustoming or, so to say, training of organisms from one group of plants on to another has to be recognized ; and, further, even though sometimes a strain of the organism does not actually form the nodules, it

[1] Revis, Cecil. *Centr. f. Bakt.*, II Abt., Bd. 31 & 34 (1911 & 1913–14); *Proc. Roy. Soc.*, B., Vol. 85 & 86 (1912 & 1913).

[2] Smith, Greig. *Proc. of the Lin. Soc.*, N. S. Wales, Vol. 31, pp. 264–294 (1906).

[3] Laurent. *Compt. Rend. Acad. Sci.*, 111, p. 754, & *Bot. Centr.*, 1891.
Mazé. *Ann. Inst. Past.*, 13, p. 145 (1899).
Moore. *U. S. Dept. Agr. Bur. Plant Indus. Bull.* 71 (1905).
Kellerman. *Centr. f. Bakt.*, II Abt., 34, pp. 42–50 (1912).

nevertheless benefits the plants, as is shown in our experiments, by fixing nitrogen and stimulating their growth. We shall not be far wrong, therefore, if we take nitrogen fixation and stimulation thereby as the essential function of the organism, and if we look upon all organisms as belonging to one species only, the production or non-production of nodules being assumed to depend on other factors such as suitable reaction of the root cell-sap and presence or absence of substances to which the organism is accustomed before isolation.

Turning to the question of nitrogen fixation by the organism, as already mentioned, a quantitative determination of the dry matter produced and the nitrogen contained in it was made in both the exp riments. The results obtained are given in the following tables.

TABLE V.

1917-18.

| Treatment | Peas (*Pisum sativum*) Dry matter in crop | % of N in dry matter | Mgm. N in dry matter | Mgm. N in seeds | Total gain in N in mgm. | Math (*Phaseolus aconitifolius*) Dry matter in crop | % of N in dry matter | Mgm. N in dry matter | Mgm. N in seeds | Total gain in N in mgm. | Gram (*Cicer arietinum*) Dry matter in crop | % of N in dry matter | Mgm. N in dry matter | Mgm. N in seeds | Total gain in N in mgm. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 2·4 | 1·41 | 33·84 | 33·20 | 0·64 | 0·5 | 1·40 | 7·00 | 4·95 | 2·05 | 1·5 | 1·07 | 16·05 | 13·10 | 2·95 |
| B | 6·3 | 1·72 | 108·36 | 33·20 | 75·16 | 3·2 | 1·93 | 61·76 | 4·95 | 56·81 | 4·5 | 1·05 | 47·25 | 13·10 | 34·15 |
| C | 4·8 | 1·51 | 72·48 | 33·20 | 39·28 | 2·5 | 2·15 | 53·75 | 4·95 | 48·80 | 4·2 | 0·90 | 37·80 | 13·10 | 24·70 |
| D | 6·1 | 1·91 | 116·51 | 33·20 | 83·31 | 3·0 | 2·32 | 69·60 | 4·95 | 64·65 | 4·4 | 1·80 | 79·20 | 13·10 | 66·10 |
| E | 5·1 | 1·90 | 96·90 | 33·20 | 63·70 | 3·4 | 2·39 | 81·26 | 4·95 | 76·31 | 4·7 | 1·92 | 90·24 | 13·10 | 77·14 |
| F | 4·8 | 1·82 | 86·56 | 33·20 | 53·36 | 2·5 | 2·21 | 55·25 | 4·95 | 50·30 | 5·1 | 2·10 | 107·10 | 13·10 | 94·00 |
| G | 3·9 | 1·95 | 76·05 | 33·20 | 42·85 | 1·9 | 2·14 | 40·66 | 4·95 | 35·75 | 3·6 | 0·94 | 33·84 | 13·10 | 20·74 |

TABLE VI

1917-18

| Treatment | Maize Dry matter in crop | % of N in dry matter | Mgm. N in dry matter | Mgm. N in seeds | Total gain in N in mgm. | Oats and Wheat Dry matter in crop | % of N in dry matter | Mgm. N in dry matter | Mgm. N in seeds | Total gain in N in mgm. |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 2·5 | 0·77 | 19·25 | 17·75 | 1·50 | 0·9 | 0·62 | 5·58 | 3·84 | 1·92 |
| B | 10·0 | 1·57 | 157·00 | 17·75 | 139·25 | 4·5 | 1·01 | 45·45 | 3·84 | 41·61 |
| C | 7·2 | 1·45 | 104·40 | 17·75 | 86·65 | 5·5 | 0·84 | 46·20 | 3·84 | 42·36 |
| D | 9·5 | 1·67 | 158·65 | 17·75 | 140·90 | 2·0 | 1·15 | 23·30 | 3·84 | 19·46 |
| E | 6·0 | 1·78 | 106·80 | 17·75 | 89·05 | 3·0 | 1·10 | 33·00 | 3·84 | 31·51 |
| F | 7·0 | 1·69 | 118·30 | 17·75 | 100·55 | 2·5 | 0·88 | 22·00 | 3·84 | 18·16 |
| G | 9·0 | 1·24 | 111·60 | 17·75 | 93·85 | 3·5 | 1·01 | 35·35 | 3·84 | 31·51 |

## TABLE VII

1918-19

| Treatment | PEAS (*Pisum sativum*) | | | | | MATH (*Phaseolus aconitifolius*) | | | | | GRAM (*Cicer arietinum*) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dry matter in crop | % N in dry crop | Mgm. N in dry matter | Mgm. N in seeds sown | Total gain of N in mgm. | Dry matter in crop | % N in dry crop | Mgm. N in dry matter | Mgm. N in seeds sown | Total gain of N in mgm. | Dry matter in crop | % N in dry crop | Mgm. N in dry matter sown | Mgm. N in seeds | Total gain of N in mgm. |
| A | 2·1 | 1·52 | 31·92 | 30·80 | 1·12 | 0·6 | 1·25 | 7·50 | 5·79 | 1·71 | 1·5 | 0·96 | 14·40 | 12·25 | 2·15 |
| B | 5·0 | 1·73 | 86·50 | 30·80 | 55·70 | 2·1 | 1·84 | 38·64 | 5·79 | 32·85 | 2·4 | 1·21 | 29·04 | 12·25 | 16·79 |
| C | 3·7 | 1·65 | 61·05 | 30·80 | 30·25 | 2·0 | 1·60 | 32·00 | 5·79 | 26·21 | 2·4 | 1·05 | 25·20 | 12·25 | 12·95 |
| D | 5·2 | 2·12 | 110·24 | 30·80 | 79·44 | 2·5 | 2·10 | 52·50 | 5·79 | 46·71 | 2·0 | 1·90 | 38·00 | 12·25 | 25·75 |
| E | 4·5 | 1·92 | 85·40 | 30·80 | 54·60 | 3·1 | 2·32 | 71·92 | 5·79 | 66·13 | 2·4 | 1·65 | 39·60 | 12·25 | 27·35 |
| F | 4·5 | 1·70 | 76·50 | 30·80 | 45·70 | 2·5 | 2·19 | 54·75 | 5·79 | 48·96 | 3·6 | 2·15 | 77·40 | 12·25 | 65·15 |
| G | 5·2 | 1·62 | 84·24 | 30·80 | 53·44 | 2·0 | 2·15 | 43·00 | 5·79 | 37·21 | 2·4 | 1·10 | 26·40 | 12·25 | 13·15 |

## TABLE VIII

1918

| Treatment | OATS (*Avena sativa*) | | | | | WHEAT (*Triticum* sp.) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dry matter in crop | % of N in dry crop | Mgm. N in dry matter | Mgm. N in seeds sown | Total gain of N in mgm. | Dry matter in crop | % of N in dry crop | Mgm. N in dry matter | Mgm. N in seeds sown | Total gain of N in mgm. |
| A | 0·7 | 0·61 | 4·27 | 2·76 | 1·51 | 0·7 | 0·91 | 6·37 | 4·48 | 1·89 |
| B | 10·2 | 0·99 | 100·98 | 2·76 | 98·22 | 5·8 | 1·16 | 67·28 | 4·48 | 62·80 |
| C | 4·5 | 0·64 | 28·80 | 2·76 | 26·04 | 2·5 | 1·05 | 26·25 | 4·48 | 21·77 |
| D | 5·8 | 1·02 | 59·16 | 2·76 | 56·40 | 2·6 | 1·15 | 29·90 | 4·48 | 25·42 |
| E | 4·9 | 1·10 | 53·90 | 2·76 | 51·14 | 2·7 | 1·10 | 29·70 | 4·48 | 25·22 |
| F | 5·9 | 0·91 | 53·69 | 2·76 | 50·93 | 2·9 | 1·12 | 32·48 | 4·48 | 28·00 |
| G | 3·7 | 0·74 | 27·38 | 2·76 | 24·62 | 2·5 | 1·03 | 25·45 | 4·48 | 20·97 |

It will be seen from these tables that different amounts of nitrogen were taken up by the plants. It is clear that compared with the nitrogen taken up by plants in the control pots more nitrogen is found in plants inoculated with the nitrogen-fixing organisms irrespective of the presence or absence of nodules. The question, therefore, arises how it could have been assimilated by the plants. In the case of the control there is little or no increase in nitrogen over that already contained in the seeds and therefore nothing is to be accounted for. In those cases where $KNO_3$ was added it is easily explained. Similarly in the case of those plants where nodules were formed this gain in nitrogen can be explained as having been due to nitrogen-fixation taking place in the nodule and its final product which is a soluble protein being passed on to the plant. In the absence of nodule formation we are led to suppose that the benefit to the plant must have been due either to (1) the organism having entered inside the root tissue without nodule formation, or (2) the nitrogen fixed in the sand becoming available to the plants.

With reference to the first supposition it has already been found in a previous experiment (*loc. cit.*) that examination of a number of sections of the root did not show any organisms inside the tissue. This time the technique was varied by plating portions of roots of plants without nodules after treating them in the same way as nodules, *i.e.*, by sterilizing the outside with mercuric chloride solution, washing them with sterile water and plating them after crushing on soil-extract-mannite-agar. No growth could be obtained on any of the plates. Simultaneously with this test same portions of roots were plated without sterilizing the outside to see whether the nodule organisms were attracted towards the roots as is generally supposed. Similarly plates were also made from the sand to see whether the organisms were still living in the sand at the end of the experiment. In both the latter cases growth of the nodule organism was obtained. As a result of all these tests it is evident that the organism is still living in the sand and, though it is closely associated with the outside of roots, it does not penetrate inside the root tissue.

We are therefore left with the other alternative supposition, *viz.*, that nitrogen might be fixed in the sand, for explanation of the results. This raises a number of questions ; first whether the nitrogen fixed by the organism in the sand is completely absorbed or some of it is left in the sand ; (2) whether the soluble protein which is supposed to be the final product of nitrogen fixation requires to be ammonified or nitrified by further bacterial action ; (3) whether the soluble protein, the final product of such fixation, could be absorbed as such by the plant ; (4) whether the process of nitrogen fixation is exactly the same in the sand as in the nodule ; and finally (5) whether some

intermediary product in the formation of soluble protein is available to the plant and can be absorbed by it. We shall attempt to answer these questions *seriatim*.

Determinations of nitrogen in the sand of the respective pots did not show any increase in nitrogen ; ammonia, nitrites and nitrates could not be detected in any of the pots. The organic nitrogen scarcely showed any appreciable increase beyond what may be accounted for as experimental error, except in the case of the azotobacter-inoculated ones. Had the nitrogen fixation gone on vigorously in the sand in the case of the nodule organism it should have been possible to find a large amount of nitrogen still remaining in the sand. No such increase is found to have taken place, which appears to suggest that the fixation of nitrogen by the organism and the assimilation by the plant go on approximately at the same rate, implying the necessity of the removal of the nitrogenous material by the plant before any further nitrogen fixation can take place. This supposition lends support to the view about the function of the host plant taken by Golding[1] who has already shown large gains of nitrogen by the root nodule organism to have taken place by filtration of the soluble products of growth which prevent the assimilation of nitrogen in artificial cultures.

Although it has not been possible to find any ammonia, nitrites or nitrates in the sand, it was suggested that ammonification or nitrification of the soluble protein may have been brought about by the root nodule organism or other organisms, implying of course infection of the sand with the latter. It was therefore proposed to separately inoculate a portion of the sand from the inoculated pots and some of the uninoculated ones in each of the following culture solutions, *viz.*, (1) peptone water in order to detect the presence of ammonifying organisms, (2) Omelianski solution so as to test the presence of nitrifying organisms. It was not considered necessary to examine all the uninoculated pots.

Even after a period of four weeks' inoculation, nitrites or nitrates could not be detected in Omelianski solution, showing the absence of nitrifying organisms or nitrification by the nodule organisms. In peptone water no ammonia was found during the first 24 hours ; after 72 hours, however, about 4 milligrams nitrogen as ammonia was found in each of the peptone flasks from the inoculated pots. Those from uninoculated pots did not show any increase. At first it was suspected that some infection might have taken place. On examining the cultures, however, there was no putrefactive smell usually associated with the ammonifiers commonly present in Pusa soil. Examination under the microscope further showed no other organism except the nodule

[1] Golding, J. *Jour. Agric. Sci.*, Vol. I, pp. 59–65 (1905-06).

organism or the azotobacter if the culture happened to be made from the azotobacter-inoculated pot. As this result was unexpected pure cultures of the nodule organisms as well as of azotobacter were inoculated in duplicate into peptone flasks and incubated at 30°C. and distilled with magnesia after three days and one week in order to find how much ammonia was formed by them. The results are tabulated below.

TABLE IX.

| | Mgm. nitrogen found as ammonia | |
|---|---|---|
| | 3 days | 1 week |
| Pea nodule organism .. .. .. | 4·2<br>4·9 | 6·3<br>4·9 |
| Math nodule organism .. .. .. | 4·9<br>3·5 | 5·6<br>4·2 |
| Gram nodule organism .. .. .. | 4·2<br>3·5 | 5·6<br>4·9 |
| Azotobacter .. .. .. .. | 3·5<br>2·8 | 4·2<br>3·5 |

From this it is clear that the nodule organism can ammonify a fraction of the peptone though it cannot at all be compared with the other ammonifiers.

It may be of interest to mention in passing that Erwin Smith[1] has been able to demonstrate ammonia production in a culture solution by a closely related organism *B. tumefaciens* which causes crown gall in plants. He has also shown that by injecting dilute ammonia in growing plants, intumescences similar to crown gall are produced, and therefore considers it probable that ammonia liberated within the cell in small quantities by the imprisoned bacteria must be one of the causes of the excessive and abnormal cell proliferation in crown gall.

Since some ammonia is formed from peptone by the nodule organism it is possible that it may also transform some of the soluble protein produced by its activity. It is equally possible for the plant to assimilate a portion of the soluble protein as such as it has been demonstrated by Hutchinson and Miller[2] that plants can absorb some complex soluble organic nitrogenous substances. It is, however, immaterial whether the plant assimilates the

[1] Smith, Erwin F. "Mechanism of Tumour growth in Crown Gall." *Journ. o Agr. Research, Washington*, Vol. VIII, No. 5, pp. 165–185.

[2] Hutchinson & Miller. *Centr. f. Bakt.*, II Abt.. 30, p. 5 (1901).

soluble protein or an ammonium compound so long as the nitrogen fixed by the organism in the sand is available to the plant in some form.

The question whether the process of nitrogen fixation is the same in the sand as in the nodule is difficult of solution as nothing is known with certainty as to how the nitrogen fixation takes place in the nodule itself beyond the well-known fact that the nodule becomes richer in nitrogen than the rest of the root.

We determined the percentage of nitrogen in the nodules and the part of the roots without nodules at different stages, and we find that the percentage of nitrogen in the nodules of sann-hemp (*Crotalaria juncea*) continually decreases from the first week onwards.

TABLE X.

| | 1st | 2nd | 3rd | 4th | 5th | 6th |
|---|---|---|---|---|---|---|
| Nitrogen in nodules, per cent. .. | 10·81 | 9·46 | 6·68 | 5·34 | 3·06 | 3·36 |
| Nitrogen in rest of root, per cent. .. | 1·95 | 1·65 | 1·45 | 1·19 | 1·14 | 1·05 |

Similar analytical results with yellow lupines were obtained by Stoklasa.[1] The present writer hopes to follow up these interesting results in the case of other plants.

Although the chemistry of the process is not known with certainty we propose to pass in rapid review some views already expressed as to how nitrogen fixation may be taking place.

These views do not refer to any particular nitrogen-fixing organism but are applicable to the general process of nitrogen fixation whether by azotobacter, Clostridium or the nodule organism. The views may be classified according to the chemical reactions on which they are based.

Thus Gautier and Drouin[2] assume oxidation of free nitrogen by the microbes to nitrous and nitric acid which would be subsequently reduced to ammonium compound and further changed to other nitrogenous substances. In support of this it may be mentioned that nitrates are sometimes found in the nodules of the leguminosæ. Gerlach and Vogel[3] hold that the free nitrogen combines directly with carbon compounds and cites the absorption of free nitrogen by carbide as an analogous process. Heinze[4] supports this view by

[1] Stoklasa. *Landw. Jahrb.*, 24, p. 827 (1895).
[2] Gautier & Drouin. *Compt. Rend. Acad. Sci.*, 106 (1888), 114 (1892).
[3] Gerlach & Vogel. *Centr. f. Bakt.*, II Abt., 9, pp. 817 & 881.
[4] Heinze. *Landw. Jahrb.*, 35 (1906).

mentioning his detection of hydrocarbons of the acetylene series in crude nitrogen-fixing cultures and makes out an equation resulting in the formation of hydrocyanic acid by the combination of acetylene and free nitrogen, but as this compound would be fatal to the cells it cannot be assumed to exist longer than a moment but is supposed to undergo immediate transformation into ammonium formate. Winogradski[1] and Stoklasa[2] assume direct synthesis of free nitrogen and nascent hydrogen into ammonia, which would in its turn be at once utilized in the process of protein or asparagin formation. Asparagin may perhaps be found in the nodules sometimes but the development of nascent hydrogen by the nodule organism with which we are chiefly concerned is not demonstrated.

It may perhaps be of interest to mention that attempts based on the chemical reactions involved in the views expressed above have already proved successful in securing free atmospheric nitrogen on a large scale independent of bacterial activity by utilizing electrical power as a source of energy. Each of the views of the biological process, therefore, has its counterpart in an industrial process. The three processes which have thus proved capable of practical application under certain limitations are (1) the arc process, (2) the cyanamide process, (3) the Haber process.

The arc process produces nitric acid by means of the combustion of nitrogen and oxygen in electric arc.

The cyanamide process depends upon the combustion of nitrogen with calcium carbide.

The Haber process involves the direct synthesis of nitrogen and hydrogen. Recently George Claude has improved the Haber process of producing ammonia synthetically by the application of high pressure.

There is yet another view held by Oscar Loew and Aso.[3] It can be represented by the following equation :—

$$N_2 + 2\,H_2O = NO_2NH_4$$

The ammonium nitrite so found is supposed to be rapidly reduced to ammonia. In support of this view the authors point out the fact that they found nitrites occurring in nodules although they were not able to detect any ammonia in them. Since then, however, Hutchinson and Miller[4] have shown the presence of ammonia in the nodules. The view taken by Loew and Aso.[5]

[1] Winogradski. *Compt. Rend., Paris,* 116 (1893), 118 (1894); *Archives des Sci. Biol., St. Petersberg,* 1895, iii, 297–352.

[2] Stoklasa. *Centr. f. Bakt.,* II Abt., 21, 484–509 & 620–632 (1908).

[3] Loew & Aso. *Bull. Col. of Agr.,* Vol. VII, pp. 567–574, 1906–1908.

[4] Hutchinson & Miller. *Centr. f. Bakt.,* II Abt., 30, p. 514 (1911).

[5] Loew & Aso. *Loc. cit.*

is further supported by the fact that Loew[1] was able to show the occurrence of the reaction at ordinary temperatures by the action of platinum black upon nitrogen in the presence of alkali. Russell and Smith[2] could not confirm this result as they did not find any such nitrite-formation in their experiments. Loew[3] thinks Russell's failure to get the nitrites formed to be due to badly prepared platinum black.

One point of agreement among all these four divergent views about nitrogen fixation is, as already observed by Loew and Aso[4] the formation of ammonia before the commencement of protein synthesis.

The chemical compounds actually found in the nodules are presumed to be a strong argument for any particular theory ; but the compounds found by different investigators have been detected in nodules of plants grown in soil. It does not necessarily follow therefore that the presence of a particular compound in the nodules means the manufacture of the compounds in the nodule by bacterial activity, as the possibility of absorption of these compounds from the soil instead of their being the product of bacterial activity is not altogether excluded. This can be proved only if the nodules under examination are got from plants grown in sterile sand freed from any of the compounds under controlled conditions as in our experiments. As the plants in our experiments were required for determining the nitrogen content we were not able to carry out the examination of the nodule contents but we hope to do this as soon as a large number of plants grown under these conditions become available.

As already mentioned in the course of previous work just described, the root nodule organism was isolated direct from the inoculated sand on soil-extract-mannite-agar. It was, therefore, thought that there should be no difficulty in isolating it direct from soil. It may be pointed out that the life-cycle of the organism from the soil through the nodule and back to the soil is shrouded in mystery and few attempts to isolate the organism from soil are recorded. While their presence in soil cannot be doubted, their isolation direct from the soil is generally not supposed to have been accomplished, as may be seen from the following remarks made by Russell[5] : " None of these organisms, however, could be found in the soil, nor indeed has any one yet succeeded in finding them there although their existence cannot be doubted."

[1] Loew. *Ber. Deutsch. Chem. Ges.*, 23, p. 447 (1890).
[2] Russel & Smith. *Jour. Agr. Science*, I, pp. 444–453 (1905).
[3] Loew. *Jour. Agr. Science*, III, p. 320 (1908–10).
[4] Loew & Aso. *Loc. cit.*
[5] Russell. " Soil conditions and plant growth," 3rd ed., 128 (1917).

Believing this statement of Russell's to be correct, Lipman and Fowler[1] communicated an article to " Science " claiming to be the first to have isolated the root nodule organism from soil by proving that the organism so isolated was capable of forming nodules on a number of legumes though not in all. They pointed out that prior to them probably only Gage[2] had mentioned direct isolation from soil. On looking over Gage's paper, however, it is not clear whether he attempted anything like direct plating from the soil emulsion or whether he enriched his culture by repeated inoculations in a liquid medium. The report of his work already indefinite on account of the incompatible results obtained is rendered more confusing on account of the unusual selection of descriptive terms. Kellerman and Leonard[3] observe with reference to Gage's work that " even if his conclusions are absolutely correct no real advance has been made in our knowledge of the life-history of *B. radicicola.*"

Previous to Lipman and Fowler's[4] paper, however, a synthetic medium for growing nodule organism was developed by Greig Smith[5] who stated that it was specific, that it was possible by its use to easily count the number of nodule organisms per gram of soil by mere plating. The medium consisted of levulose, asparagin and citrates of sodium and potassium (for actual amounts of different components, see later) and was tested in this laboratory soon after the appearance of Greig Smith's paper. It was found then that although the medium could not be said to be specific, yet large numbers of colonies of the nodule-forming organism were found growing on it in the plates.

Lipman and Fowler,[6] however, put aside Greig Smith's claim to having discovered a specific medium for *B. radicicola* chiefly relying on Kellerman and Leonard's work. The last named investigators could not find experimental evidence to confirm Greig Smith's results and were themselves unable to isolate the organism from soil on other media except in one case where a pure culture had been previously introduced into the soil.

Löhnis has, however, already pointed out that Beijerinck and Nobbe and his associates had isolated the organism from the soil and there is no more any question of priority about the work of Lipman and Fowler[7] who have accepted Löhnis' statement so far as the work of Nobbe and his associates is concerned.

[1] Lipman & Fowler. *Science*, n. s., 41, pp. 256–259 (1915).
[2] Gage. *Centr. f. Bakt.*, II Abt., 27, pp. 7–48 (1910).
[3] Kellerman & Leonard. *Science*, n. s., 38, p. 95 (1913).
[4] Lipman & Fowler. *Loc. cit.*
[5] Greig Smith. *Centr. f. Bakt.*, II Abt., 34, pp. 227–229 (1912).
[6] Lipman & Fowler. *Science*, n. s., 41, p. 725.
[7] Lipman & Fowler. *Loc. cit.*

Leaving aside the question of priority therefore, we would like to take this opportunity of pointing out that the chief reason why the legume nodule organism is not recognized to have been isolated from the soil is that the question has not been thoroughly examined ; statements to the effect that nodule organism does not generally grow in ordinary nutrient media are unquestionably accepted as correct ; and therefore an organism, which there might be some reason for believing to be the nodule organism, is rather assumed to be different from it because there is little inclination to question a statement so authoritatively made. This happens, the more so, because of another statement being accepted as correct, *viz.*, that the organism fixes considerable quantities of nitrogen in culture flasks. Thus when any organism closely resembling the nodule organism in other respects is found not to fix nitrogen in large quantities in the laboratory the natural tendency is to take it as different from the latter.

Thus a question arose in this laboratory whether a certain organism was a nodule organism or not. The organism in question was isolated from Pusa soil on Lipman & Brown's synthetic agar. Instead of actually putting it down as a legume nodule organism it was subsequently described as similar to the nodule organism as follows[1] :—" Short rods forming white, slimy, tenacious colonies, and similar in morphological characters to root nodule organisms of *Dolichos lablab*, subsequently gave similar cultural characters on ash maltose agar." The chief reason for doing so was that the organism under examination fixed only a small quantity of nitrogen, about 1 to 2 mgm. per 100 c.c. of the culture in the laboratory, and inoculation tests were not considered necessary when nitrogen fixation was so small. When, however, the nitrogen fixation effected by several strains of the legume nodule organism was found to be not more than 2 mgm., re-examination by inoculation tests of the question whether the organism thus isolated on Lipman and Brown's[2] synthetic agar is the legume nodule organism suggested itself ; and it was proposed to isolate from Pusa soil the organism previously described. Side by side with this experiment it was proposed to compare other media used and recommended by Greig Smith[3] and Lipman and Fowler[4] for isolating the root nodule organism from the soil. Soil-extract-mannite-agar, the medium used to isolate organisms from nodules in our work, was also included for comparison.

[1] Hutchinson. *Mem. Dept. Agr. India, Bact. Ser.*, I, p. 9.
[2] Lipman & Brown. *Centr. f. Bakt.*, II Abt., 25, p. 447.
[3] Greig Smith. *Loc. cit.*
[4] Lipman & Fowler. *Loc. cit.*

It may perhaps be an advantage if the composition of the different media is set down for easy reference.

| Synthetic agar | Levulose agar | Maltose agar | Soil-extract-mannite-agar |
|---|---|---|---|
| 10·0 Dextrose | 20·0 Levulose | 10·0 Maltose | 20·0 Mannite |
| 0·5 $K_2HPO_4$ | 0·6 Asparagin | 1000·0 Soil extract | 0·5 $K_2HPO_4$ |
| 0·2 $MgSO_4$ | 1·0 Sodium citrate | 20·0 Agar | 1000·0 Soil extract |
| 0·05 $KNO_3$ | 1·0 Potassium citrate | | 20·0 Agar |
| 20·0 Agar | 20·0 Agar | | |
| 1000·0 Distilled water | 1000·0 Distilled water | | |

Pusa soil (dilution 1 : 10,000) was plated on all these four media. Such colonies as have the characteristic appearance of the nodule organism, were selected and examined under the microscope. The organisms had the same morphological characters as noticed in the case of the nodule organisms. Pure cultures were made from two colonies from each plate. These cultures were inoculated on the following plants: Sann-hemp (*Crotalaria juncea*), Arhar (*Cajanus indicus*), Cow-pea (*Vigna catjang*) and Math (*Phaseolus aconitifolius*).

The plants were grown in pots in sterile sand as in previous experiments and all of them showed nodule formation when they were uprooted at the end of the experiment. This shows that the root nodule organisms can be isolated direct from the soil on these media. It may be mentioned that other kinds of colonies also grew on all the plates and none of the media tried can be called absolutely specific, but in order to test which of these gives the highest number or a proportionately higher number of nodule organisms of the total number, the following experiment was devised.

Three flower pots were filled with Pusa soil. Two legumes, sann-hemp and Math, were grown in one. Two cereals, oats and maize, were grown in another. The third pot was left as control. When the crops were about two weeks old, samples of these soils were taken for plating. The same four media as in the previous experiment were used. The total number of colonies and of all organism colonies of the nodule organism in each plate were counted at the end of 3 and 7 days' incubation at 30°C. and 20°C. The results are given in the following table.

TABLE XI.

| Treatment | | Soil-extract-mannite-agar | | Lipman's soil-extract-maltose-agar | | Lipman & Brown's synthetic agar | | Greig Smith's levulose agar | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of colonies after 3 days | No. of colonies after 7 days | No. of colonies after 3 days | No. of colonies after 7 days | No. of colonies after 3 days | No. of colonies after 7 days | No. of colonies after 3 days | No. of colonies after 7 days | |
| Pusa soil (control) | Total No. of colonies . | 6,500,000 | 10,000,000 | 4,300,000 | 6,200,000 | 2,100,000 | 3,300,000 | 1,400,000 | 1,800,000 | Incubated. at 20°C. |
| | Nodule organism colonies . | 500,000 | 800,000 | 100,000 | 200,000 | 100,000 | 200,000 | 100,000 | 200,000 | |
| Pusa soil (with legumes growing) | Total No. of colonies . | 21,000,000 | 30,000,000 | 7,500,000 | 10,200,000 | 4,200,000 | 6,100,000 | 2,200,000 | 3,200,000 | Incubated at 20°C. |
| | Nodule organism colonies . | 2,500,000 | 4,500,000 | 300,000 | 500,000 | 200,000 | 300,000 | 200,000 | 400,000 | |
| Pusa soil (with cereals growing ) | Total No. of colonies . | 15,000,000 | 20,500,000 | 15,000,000 | 20,000,000 | 2,200,000 | 2,800,000 | 2,100,000 | 3,200,000 | Incubated at 20°C. |
| | Nodule organism colonies . | 1,300,000 | 2,100,000 | 400,000 | 600,000 | 100,000 | 200,000 | 100,000 | 200,000 | |
| Pusa soil (control) | Total No. of colonies . | 20,000,000 | 32,000,000 | 18,000,000 | 24,000,000 | 2,000,000 | 6,800,000 | 1,500,000 | 2,500,000 | Incubated at 30°C. |
| | Nodule organism colonies . | 1,000,000 | 1,600,000 | 400,000 | 500,000 | 200,000 | 300,000 | 100,000 | 200,000 | |
| Pusa soil (with legumes growing) | Total No. of colonies . | 70,000,000 | 110,000,000 | 24,000,000 | 38,500,000 | 7,700,000 | 11,200,000 | 6,300,000 | 7,200,000 | Incubated at 30°C. |
| | Nodule organism colonies . | 9,500,000 | 15,000,000 | 1,500,000 | 2,500,000 | 200,000 | 500,000 | 700,000 | 900,000 | |
| Pusa soil (with cereals growing) | Total No. of colonies . | 30,000,000 | 45,000,000 | 20,500,000 | 31,000,000 | 5,200,000 | 8,100,000 | 7,500,000 | 10,500,000 | Incubated at 30°C. |
| | Nodule organism colonies . | 2,400,000 | 3,000,000 | 1,200,000 | 1,600,000 | 100,000 | 300,000 | 500,000 | 700,000 | |

From the above it will be seen that the number of colonies of the nodule organisms is higher in soil-extract-mannite-agar and the ratio of these colonies to others is also higher in the same medium. The minimum number of colonies per gram of dry soil is 100,000 and the maximum number is 15,000,000.

It may be mentioned that azotobacter and yeast colonies were growing on some of these plates which very much resembled the nodule organism colonies. These are excluded in our countings by examining the morphological characters of the organism in the colonies under the microscope. Further trials with these media were made in order to see whether any of them is best for isolating organisms from root nodules without sterilizing the nodules in the outside. Nodules of Val (*Dolichos lablab*), Indigo (*Indigofera arrecta*) and Cow-pea (*Vigna catjang*) were taken in separate dishes and crushed and a loopful of the emulsion thus made was separately inoculated in these media. Duplicate plates were poured from each of the dishes. The plates were incubated at 30°C. and 20°C. The examination of the plates showed that all the plates grew the nodule organism colonies, the highest number being in the soil-extract-mannite-agar and the lowest in the levulose agar of Greig Smith ; but while all the other plates showed contamination the levulose agar was particularly free from any extraneous organisms. It may be mentioned here that in one plate of levulose agar there was no growth at all, while the duplicate showed a fair number of colonies. We think this was due to the greater amount of alkali added by mistake to the plate in which no growth appeared at the time of plating. In our opinion this probably indicates that the specific action of the medium is due rather to the alkalinity of the medium than to the kind of sugar employed ; and that this is brought about by inhibiting the growth of the organisms sensitive to the higher degree of alkalinity attained by adding to the medium, at the time of plating, a few drops of the alkali the addition of which does not seem to affect the nodule organisms up to a certain point beyond which the growth of the nodule organism is also inhibited as noticed above in one of the plates. The point will be further tested with a view to find out whether the soil-extract-mannite-agar, which gives vigorous growth of the organism, can not be made specific for the nodule organism by suitable modifications, such as the addition of asparagin and a few drops of sodium carbonate solution to alter its reaction.

Pure cultures of nodule organisms from six plates were next inoculated on slants of these four media to see their effect on the growth of the organism. Judging by the amount of growth, soil-extract-mannite-agar was the best ;

next to it comes soil-extract-maltose, agar then synthetic agar (dextrose) while levulose agar was the last.

As these media differ in the kinds and amounts of sugars added it was proposed to see the effect of these sugars when 1 per cent. of each is added to Pusa soil extract with potassium phosphate. Several strains of nodule organisms were inoculated on agars prepared in this way.

The results of growth are given in the following table.

+++ = shows the most vigorous growth.
++ = shows moderate growth.
+ = shows poor growth.

TABLE XII.

| | Pea | Val | Math | Indigo | Sann-hemp | Cow-pea |
|---|---|---|---|---|---|---|
| Sucrose agar .. | ++ | +++ | +++ | +++ | +++ | +++ |
| Glucose agar .. | + | +++ | +++ | +++ | +++ | +++ |
| Lactose agar .. | + | ++ | + | + | ++ | ++ |
| Levulose agar .. | ++ | +++ | ++ | ++ | ++ | +++ |
| Maltose agar .. | ++ | +++ | +++ | ++ | ++ | +++ |
| Mannite agar .. | +++ | +++ | +++ | +++ | +++ | +++ |

From this it is evident that with mannite, glucose, maltose and sucrose the organisms give fairly equal amount of growth ; with lactose all organisms give comparatively poor growth. How far any of these sugars are useful in fixing nitrogen, however, remains to be seen, as it is possible that the greater amount of vegetative growth need not necessarily mean proportionately larger amount of nitrogen fixed. In other words, the function of nitrogen fixation may be imagined to be independent of the vegetative growth of the culture.

## SUMMARY.

1. The cross-inoculation results of the experiments reported here tend to indicate that there is only one single species of the legume nodule organism, if we take nitrogen fixation and stimulation thereby as the essential function of the organism. If, however, nodule formation is taken as the sole physiological test for distinguishing the species, then naturally the above conclusion will have to be modified.

2. Where the inoculations of the organism did not lead to nodule formation, the plants were found to derive some benefit by the nitrogen fixed by the organism. No doubt the plants with nodules have the further advantage of quickly assimilating products of metabolism of organisms growing in the nodules.

3. The root nodule organism is found to exert a beneficial influence on graminaceous plants also.

4. If the organism is growing in a porous cylinder in the centre of the glazed earthenware pot, the soluble products passing through the porous walls of the cylinder are found to benefit the plants growing outside the porous pot.

5. The chief function of the root nodule organism appears to be nitrogen fixation whether within the nodules or outside.

6. Inoculation of azotobacter gives results similar to those obtained in the case of the nodule organism when it does not form nodules.

7. The fact that there is no residual nitrogen in the case of the nodule organism as contrasted with azotobacter suggests that the fixation of the nitrogen by the organism and assimilation of the same by the plant with and without nodule formation go on approximately at the same rate; which implies the necessity of the removal of the products of metabolism by the plant before further nitrogen fixation by the organism can take place. Further experiments are necessary to furnish final evidence on this point.

8. The root nodule organism can be isolated on some media direct from the soil. Soil-extract-mannite-agar has proved so far the best. A modification of the medium so as to make it more suitable for the growth of the root nodule organism is suggested for further trial.

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